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Lysosomal Changes in Renal Proximal Tubular Epithelial Cells of Male Sprague Dawley Rats Following Decalin Exposure*

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ABSTRACT

A histochemical stain for acid phosphatase served as a marker for lysosomal alterations in renal tubular cells associated with male rat hyaline droplet nephropathy. Morphometric analysis and quantitative histochemistry were used to compare the size and acid phosphatase stain reaction of lysosomes in tubular epithelial cells of treated and control animals. Decalin exposure increased the size and significantly ($p < 0.01$) reduced the acid phosphatase stain intensity of individual lysosomes. However, there was no significant different ($p > 0.05$) between the acid phosphatase stain intensity of treated and control animals when analyzed on a whole cell basis. The increase in size of the lysosomes without a proportional increase in whole cell acid phosphatase stain intensity indicates a dilution or a failure to accommodate in the acid phosphatase concentration (stain intensity/μm^2) per lysosome. All acid phosphatase stain reaction product was contained within intact lysosomes, mitigating against the hypothesis of lysosomal enzyme leakage as the cause of cell death in decalin-induced alpha 2U globulin nephropathy.

Keywords. Hydrocarbon nephropathy; alpha 2U globulin nephropathy; alpha 2U globulin; acid phosphatase; histochemistry; histomorphometry

INTRODUCTION

Physiologic proteinuria is a characteristic feature of young adult male rats which may be associated with an androgen-dependent inefficiency of renal protein reabsorption and catabolism (1, 3). The major component of the proteinuria is a low molecular weight glycoprotein called alpha 2U globulin (1). The principal cytoplasmic organelle involved with alpha 2U globulin metabolism is the lysosome (21).

The physiologic proteinuria and metabolism of alpha 2U globulin have been associated with a potentially unique susceptibility of male rats to specific xenobiotic-induced nephrotoxicity (2, 8). Exposure of male rats to compounds such as d-limonene (23), decalin (1), 2,2,4-trimethylpentane (13, 15, 18), and JP-5 jet fuel (4) results in pathologic renal alteration. Species such as mice, dogs, and monkeys (which lack urinary alpha 2U globulin) show no evidence of nephropathy following exposure to the above compounds (1).

Excessive hyaline droplet formation in the proximal tubular epithelial cell is the primary feature of hydrocarbon-induced male rat nephropathy. The hyaline droplets are believed to reflect an abnormality in renal lysosomal catabolism (2, 16, 21). Although several studies have suggested a causal relationship between excessive hyaline droplet formation, phagolysosomal protein accumulation, and tubular epithelial cell death, the mechanism of cell death is unknown (12, 21). The present study was designed to use acid phosphatase as a biomarker to further characterize lysosomal alterations associated with hydrocarbon-induced hyaline droplet formation and renal tubular cell death.

MATERIALS AND METHODS

Laboratory Animals

Male Sprague-Dawley rats (Charles River Laboratories) were approximately 80-days-old at the beginning of the experiment. The rats were housed in stainless steel cages suspended over absorbent paper. The ambient temperature was maintained at 24°C ± 2°C and light was regulated on a 12-hr-light/dark cycle. Pelleted food (Purina Rat Chow) and

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water were provided free choice. All animals were housed, maintained, and used in accordance with DHHS (NIH) Publication 85–23 entitled "Guide for Care and Use of Laboratory Animals," and the Animal Welfare Act of 1966, as amended.

Animal Treatment

Fourteen male rats were randomly assigned to control and treatment groups. Control animals received 3 daily gavage doses of 5 ml/kg corn oil. Treatment animals received an identical dose containing 40 mg decalin/ml corn oil.

Tissue Collection and Processing

Twenty-four hr after the final dose of corn oil or corn oil/decalin the animals were anesthetized (sodium pentobarbital, 40 mg/kg) and prepared for tissue perfusion. Tissue perfusion and fixation were performed using the procedure described by Straus (20). The abdominal aorta was catheterized to allow sequential retrograde perfusion of the kidneys with 0.9% saline and 4% glutaraldehyde/0.8% NaCl/0.02 molar cacodylate buffer (pH 7.0). The kidneys were removed, sliced into thin sections and post-fixed in 4% glutaraldehyde/4% formalin/30% sucrose/0.02 molar cacodylate buffer (pH 7.0). The kidney samples were finally washed in a 30% sucrose/0.001 molar cacodylate buffer (pH 7.0) and quenched in a freezing hexane bath. The samples were then stored at −80°C until further processing.

Kidney tissue for electron microscopy was thawed and processed through 1% OsO_4, 2% uranyl acetate, and a graded series of ethanols before embedding in Lx112 epoxy (Ladd Research). Seventy nanometer sections were made using a diamond knife on a RMC ultramicrotome (Research Manufacturing Company). Electron microscopy was performed using a Joel 100Cx transmission electron microscope (80 Kv, objective aperture = 3, spot size = 2).

Kidney tissue for light microscopy was equilibrated to cryotome cabinet temperature for 30 min before sectioning. Four micrometer sections were cut with a cryomicrotome at −18°C and mounted on glass slides which had been acid cleaned and coated with an aqueous solution of 0.1% gelatin (100 bloom) and 0.01% chromium potassium sulfate. The sections were post-fixed in 4% glutaraldehyde/0.001 molar cacodylate buffer (pH 7.0) in preparation for morphometric and histochemical analysis.

Morphometric and Histochemical Analysis of Lysosomes

The diameter of individual lysosomes was estimated using an eyepiece micrometer calibrated with a reference stage micrometer (Wild-Leitz USA). Mean lysosome diameters were calculated from 5 lysosomes/tubule × 10 tubules/animal × 7 animals/group (n = 350). Acid phosphatase was detected using the naphthol AS-TR phosphate pararosaniline technique of Straus (20). Naphthol AS-TR phosphate, pararosaniline, and N,N-dimethylformamide reagents were purchased from Sigma Chemical. Quantitative histochemistry was performed using a Leitz Orthoplan light microscope equipped with a MPV2 micro-densitometer and an interference graduated filter (Wild-Leitz USA). The absorption maximum for naphthol AS-TR phosphate pararosaniline stained sections was determined to be 490 nm and all densitometric data was collected at this wavelength. Absorbance readings were recorded to estimate the acid phosphatase stain product: 1) within a given lysosome (1 μm^2 area) and 2) within a given proximal epithelial cell (100 μm^2 area). The mean absorbance (O.D.) for the acid phosphatase stain product was calculated from measurements of 10 cells/tubule × 10 tubules/animal × 7 animals/group (n = 700). Histochemical data was analyzed using a nested ANOVA procedure.

RESULTS

Renal Tubular Epithelial Cell Morphology

Normal renal tubular cell morphology and acid phosphatase stain reaction are shown in Fig. 1. Morphologic features of the acute renal tubular response to decalin exposure included: lack of an inflammatory infiltrate, diffuse involvement of single cells with sloughing into the uriniferous space, cytoplasmic condensation, and the maintenance of nuclear and lysosomal integrity (Figs. 2 and 3).

Lysosomal Morphology

All acid phosphatase stain reaction product was contained within intact lysosomes as judged by oil immersion light microscopy (1000× magnification; Figs. 1 and 2). The mean lysosome diameter of control animals was 1.2 ± 0.5 μm. The mean lysosome diameter of treatment animals was 3.5 ± 1.3 μm. Electron microscopy further confirmed the presence of enlarged, intact lysosomes in the decalin-treated animals (Fig. 3).

Acid Phosphatase Stain Reaction

The optical density (O.D.) of the acid phosphatase stain reaction was determined at the level of the whole cell (100 μm^2) and the individual lysosome (1 μm^2) (Fig. 4). The mean whole cell O.D. value for control animals was 0.217 ± 0.042. The mean whole cell O.D. value for treatment animals was

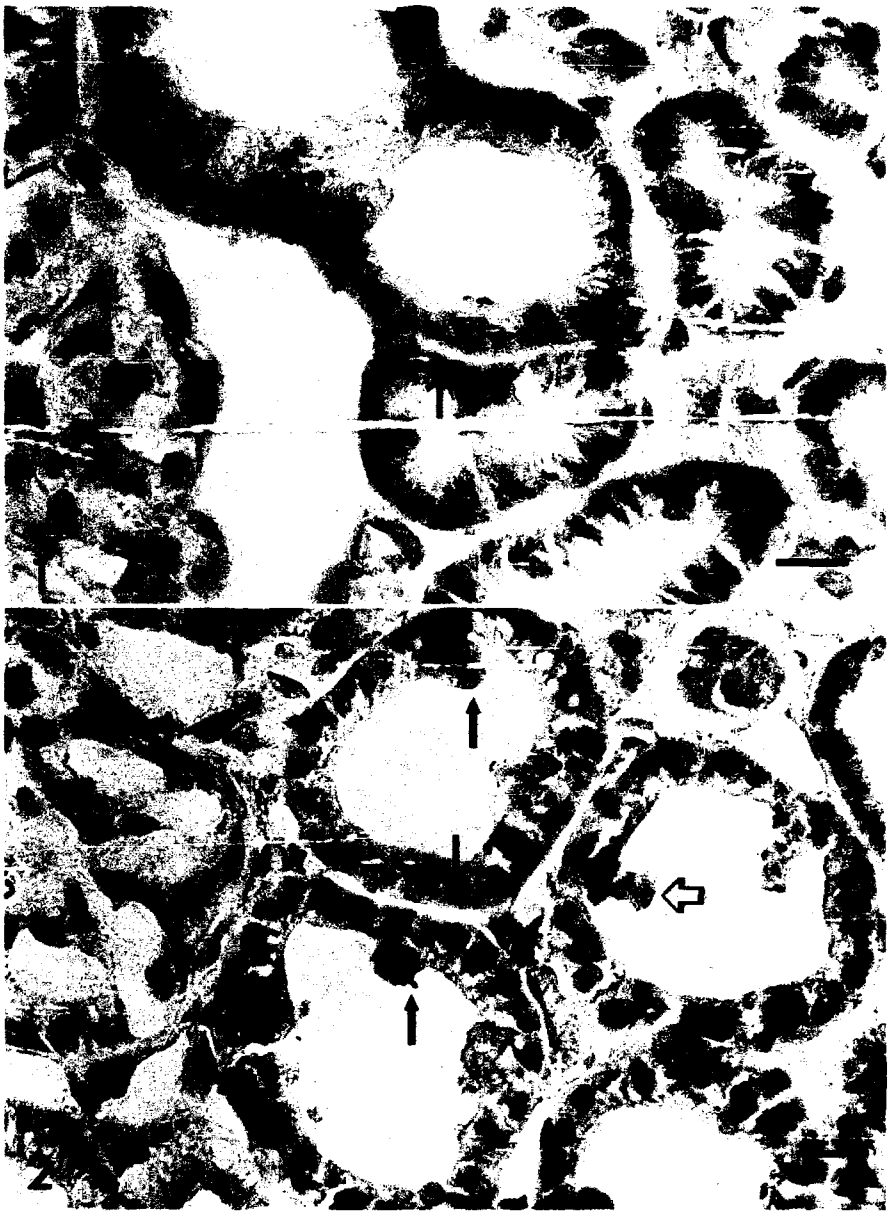

FIG. 1.—Frozen section of renal tubular epithelium from a control animal. Note small, dense, red-staining lysosomes (→). (Original magnification ×1,000.) Bar = 10 μm.

FIG. 2.—Frozen section of renal tubular epithelium from a decalin-treated animal. Note large, pale, red-staining lysosome (→). An exfoliated epithelial cell can be seen in the tubular lumen containing large, intact lysosomes (⇨). Note cytoplasmic condensation of renal tubular cells undergoing early degenerative changes (➡). (Original magnification ×1,000.) Bar = 10 μm.

0.211 ± 0.043. The mean individual lysosome O.D. value for control animals was 0.684 ± 0.083. The mean individual lysosome O.D. value for treatment animals was 0.298 ± 0.064.

Statistical Analysis

A nested ANOVA procedure was used in the data analysis to detect any subordinate influence of treatment effect and animal source on tubule choice. Within a given experimental group (control or treatment), there was no significant difference ($p > 0.05$) in the acid phosphatase data obtained from a given proximal tubule for either the individual lysosome or whole cell analysis. However, there was a significant difference ($p < 0.05$) obtained at the animal level within a given experimental group for both the

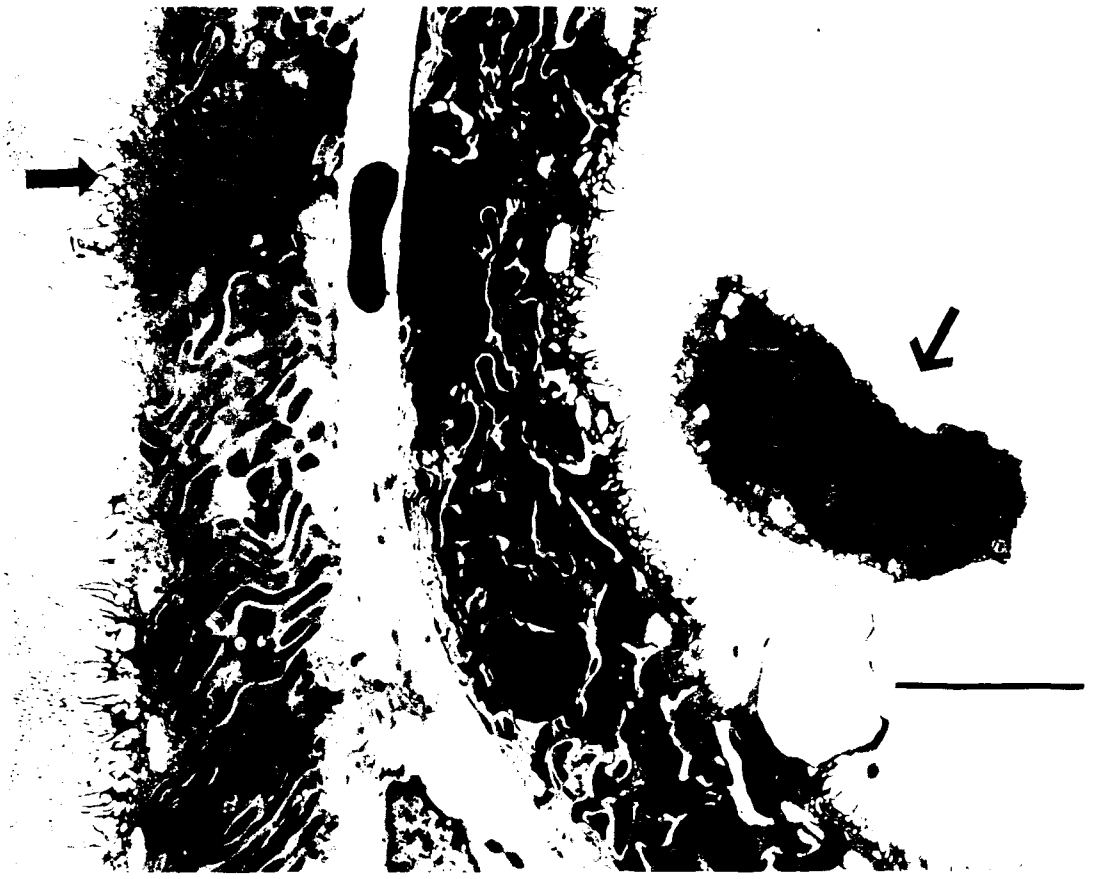

FIG. 3.—Electron micrograph of renal tubular epithelium from a decalin-treated animal. The tubular lumen on the right half of the photomicrograph contains an exfoliated epithelial cell (→) with enlarged, intact lysosomes. The tubule on the left half of the photomicrograph contains an epithelial cell in the early stages of cytoplasmic condensation (➧). (Original magnification ×5,200.) Bar = 5 μm.

individual lysosome and whole cell analysis. We attribute the animal level interaction to represent biological variation.

On a whole cell basis there was no significant difference ($p > 0.05$) between the acid phosphatase data of control and treatment groups. However, there was a significant difference ($p < 0.01$) between the acid phosphatase data of experimental groups when analyzed on the basis of the individual lysosome (Fig. 4).

DISCUSSION

The histomorphometric techniques presented in this study are useful biomarkers of decalin exposure and provide a direct method for the comparison of morphologic and biochemical alterations associated with hyaline droplet nephropathy. This differs from previous studies where morphologic parameters were based on non-specific protein stains (e.g., hematoxylin and eosin, Mallory's Heidenhain, or Lee's methylene blue-basic fuchsin) and biochemical data were obtained by indirect methods such as tissue homogenization (5, 8, 12, 14, 17). The acid phosphatase stain has an advantage over non-specific protein stains, because it specifically reacts with a membrane-bound lysosomal enzyme and produces a distinct color reaction with minimal background staining (Figs. 1 and 2).

The histomorphometric data for lysosomal acid phosphatase obtained following acute decalin exposure differed depending upon the tissue area being analyzed. There was no significant difference ($p > 0.05$) in the enzyme reaction between control and treated animals when analyzed on a whole cell basis. However, there was a significant decrease ($p < 0.01$) in the enzyme reaction of individual affected lysosomes (Fig. 4).

The lack of a significant difference in the whole cell acid phosphatase stain reaction between control animals and animals demonstrating hyaline droplet nephropathy agrees with previous studies based on the enzymatic analysis of tissue homogenates (14, 17). However, increased lysosomal size without a proportional increase in whole cell acid phosphatase stain reaction indicates a dilution or a failure to accommodate in the acid phosphatase content (stain

intensity/μm^2) of individual affected lysosomes-phagolysosomes. Enzymatic dilution of individual lysosomes-phagolysosomes could be important in regards to the effect of decalin exposure on the lysosomal processing of proteins.

Current models of xenobiotic-induced hyaline droplet nephropathy directly associate agent exposure to elevated levels of chemically modified alpha 2U globulin, renal tubular hyaline droplet formation, and cell death (11, 19, 21). Studies with unleaded gasoline and 2,2,4-trimethylpentane have shown that renal areas with the highest mitotic activity also have the highest hyaline droplet formation and greatest amount of renal tubular cell death (19). The reversible binding of hydrocarbon metabolites to alpha 2U globulin has been implicated in reducing the catabolism of the protein and increasing the accumulation of alpha 2U globulin in the tubular epithelial cell (11, 21). Although it is not known how excessive protein accumulation results in renal tubular cell death, it has been suggested to occur through an autolytic process induced by lysosomal enzyme leakage (12, 19).

In the present study, the enlarged lysosomes-phagolysosomes induced by decalin exposure are clearly intact in the acid phosphatase stained sections (Fig. 2). In fact, damaged renal tubular cells which have been released into the tubular lumen have well preserved nuclear morphology and intact lysosomes-phagolysosomes (Figs. 2 and 3). This data suggests that the cell death induced by decalin exposure occurred by a non-autolytic mechanism.

Cell death can be divided into 2 morphologically and biochemically distinct categories: necrosis and apoptosis (9, 22, 24). Necrosis is a degenerative, autolytic process that follows irreversible cell injury. Apoptosis is an inherent, programmed process of senescent elimination occurring normally in all tissues (24). Necrosis typically is not limited to a single cell, but involves contiguous parenchymal cells and inflammatory cells. Morphologic changes which characterize cellular necrosis include marginal clumping of loosely textured nuclear chromatin, cytoplasmic swelling, and lysosomal lysis (22). Apoptosis usually affects scattered single cells with no evidence of inflammatory infiltration. Apoptosis is involved in the tissue transformations which occur during embryogenesis and the normal cell turnover associated with tissue homeostasis (22). Morphologic changes which characterize the process of apoptosis include aggregated granular chromatin abutting the nuclear membrane, condensation of the cytoplasm, maintenance of the integrity of cellular organelles (including lysosomes), and formation of blunt protuberances on the cell surface which separate to become apoptotic bodies (22).

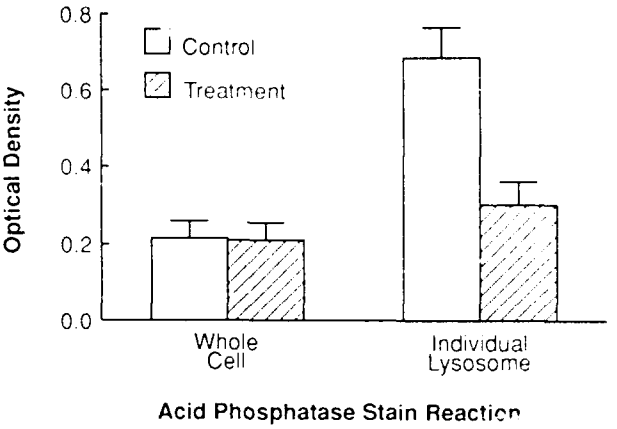

FIG. 4.—Changes in the acid phosphatase stain reaction of renal tubular epithelial cells following decalin exposure. Bar graphs represent the mean optical density ±1 standard deviation.

Pathologic conditions can accelerate the process of apoptosis and bring about the selective elimination of critically damaged cells (7). Although there have been a limited number of studies regarding the role of apoptosis in homeostatic or pathologic renal processes, accelerated apoptosis has been described in renal tissue with hydronephrosis (6), during the regression of hyperplasia (10), and following X-irradiation (7).

The lack of an inflammatory infiltrate, the condensation of renal tubular cell cytoplasm, and the maintenance of lysosomal integrity seen in renal sections from treated animals (Figs. 2 and 3) suggest that accelerated apoptosis may be involved in the decalin-induced nephrotoxic process. However, electron microscopic studies of renal tissue from treated animals (Fig. 3) did not reveal classical apoptotic changes (e.g., apoptotic bodies, aggregated granular chromatin abutting the nuclear membrane, abnormal convolution of the nuclear outline). This suggests that hydrocarbon-induced nephrotoxicity in the male rat is a process which may not be fully defined by the classical features of apoptosis. Further studies of homeostatic and accelerated renal apoptosis are necessary to determine if hydrocarbon-induced nephrotoxicity fits within an extended view of apoptosis or is representative of an intermediate morphologic category of cell death.

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